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Expression of an extended HMW subunit in transgenic wheat and the effect on dough mixing properties

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Abstract

Wheat line L88-31 was transformed with a gene encoding an extended form of subunit 1Dx5 to study the relationship between subunit size and the effect on dough mixing properties. Four transgenic lines were recovered, one of which expressed a truncated form of the protein with mobility between those of the wild type and extended subunits. Comparison of the Mixograph profiles and gluten protein compositions with those of the control lines and a line expressing the wild type subunit 1Dx5 transgene showed that two of the transgenic lines had poor mixing properties and that this was associated with co-suppression of HMW subunit gene expression. The other two transgenic lines had improved mixing properties (measured as increased mixing time) and this was associated with increased proportions of large glutenin polymers. None of the transgenic lines expressing the extended form of the 1Dx5 subunit showed the 'overstrong' mixing properties exhibited by transgenic lines expressing the wild type 1Dx5 transgene.

Keywords: Genetic transformation; HMW subunit; Dough mixing; Glutenin polymers

1. Introduction

The association of allelic variation in the composition of the high molecular weight (HMW) subunits of wheat glutenin with differences in dough strength is well established and the HMW subunits are widely used as predictors of quality in plant breeding programmes (Payne, 1987; Shewry et al., 2003). This association has stimulated a range of studies of the HMW subunits at the genetic, biochemical and biophysical levels (as reviewed by Shewry et al., 2003) which have indicated that several factors may be important. The first of these is variation in the total amount of HMW subunit protein resulting from the expression of three, four or five HMW subunit genes in different cultivars of bread wheat. The second factor is differences in the structures and properties of the subunits encoded by allelic genes in different cultivars. These may include the presence of additional cysteine residues, as in subunit 1Dx5, which may lead to more highly cross-linked and hence more elastic gluten (Anderson et al., 1989). However, comparison of the effects of a range of purified (i.e. from flour) and recombinant (i.e. expressed in *E. coli*) subunits on the dough mixing properties when incorporated using a reduction/reoxidation procedure and 2 g Mixograph

Abbreviations CTAB, cetyltriethylammonium bromide; DIG, digoxigenin; HMW, high molecular weight; MT, mixing time in seconds; NIR, near infra-red; PCR, polymerase chain reaction; PR, peak resistance in arbitrary units; RBD, resistance breakdown; RP-HPLC, reversed-phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl-sulphate polyacrylamide gel electrophoresis; SE-HPLC, size-exclusion high performance liquid chromatography; %UPP, percentage of unextractable polymeric protein..

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has also demonstrated a clear correlation between subunit size and dough strength, with longer proteins being more effective (Anderson et al., 1996).

To study the relationship between HMW subunit length and dough strength, we have mutated the gene encoding subunit 1Dx5 to extend the repetitive domain by 157 amino acids. This mutated gene has then been expressed in transgenic wheat and the impact on dough strength compared with that of the wild type gene.

2. Experimental

2.1. Production of transgenic wheat constructs

The pET3a-Glu-1Dx5-R853 (D'Ovidio et al., 1997) and the pLRPT (kindly provided by Dr Lee Rooke, Rothamsted Research, UK) clones were used to prepare the expression constructs pLRPT-R853. The pET3a-Glu-1Dx5-R853 clone contains the mature coding region of the 1Dx5 gene with a repetitive domain 22.5% larger of the native 1Dx5 gene (D'Ovidio et al., 1997). The pLRPT clone contains 1.3 kb of the 5′ flanking region of the Dx5 gene.

The pLRPT-R853 clone was prepared by performing several intermediate steps aimed at including the leader sequence and the 3′ flanking region of the 1Dx5 gene, both lacking in pET3a-Glu-1Dx5-R853. The addition of the leader sequence was achieved by performing a PCR amplification of the 1Dx5 gene from −128 bp (from the ATG) to 366 bp within the *N*-terminal region. To facilitate the cloning steps, the forward primer was selected to include the *Nco*I site present in the 5′ flanking region (5′-tgcacgcag*ccatgg*tcctgaa-3′) and the reverse primer was designated to have a *Bam*HI site (5′-tggata*ggatcc*aacctgctgcggacaag-3′). The expected amplicon was double digested with *Nco*I and *Bam*HI, and inserted into the *Nco*I/*Bam*HI sites of pLRPT. The resulting clone, named pLRPT*, was used in the subsequent step to insert the mature coding region of the pET3a-Glu-1Dx5-R853 (the *Stu*I/*Bam*HI fragment spanning from amino acid 4 up to the stop codon) into its *Stu*I and *Bam*HI sites. Finally, the 3′ flanking region was included in this clone by exchanging its terminal *Xcm*I/*Bam*HI fragment (spanning from amino acid 790 up to the stop codon) with the corresponding *Xcm*I/*Hin*dIII fragment of the native 1Dx5 gene that also contains 132 bp of the 3′ flanking region, including the poly A+ signal (Anderson et al., 1989). The correctness of the recombinant clones was verified by restriction digestion and nucleotide sequence analysis.

2.2. Plant material

Bread wheat (*Triticum aestivum* L.) lines L88-6 and L88-31 are spring-type genotypes that differ in their HMW glutenin subunit composition (Lawrence et al., 1988). Plants for transformation were grown, 5 per 20 cm diameter pot, in an environmentally-controlled containment glasshouse with air temperatures of 18 °C/15 °C (day/night), a relative humidity of 50–70% under ca. 350 μmol/(m^2/s) irradiance with a photoperiod of 16 h. The production of the transgenic line B72-8-12 used in this study has been reported previously by Barro et al. (1997).

2.3. Transformation

Immature scutella isolated from seeds at approximately 14–16 days post-anthesis were co-transformed using the PDS1000/He micro-projectile bombardment device (BioRad, Hemel Hempstead, UK) with the plasmids pLRPT-1Dx5-R853 and pCaIneo containing the selectable marker gene *npt*II (Muller et al., 1996) at a 1:1 molar ratio.

Plants were recovered via in vitro tissue culture using G418 selection following protocols developed by Barcelo and Lazzeri (1995), modified by Pastori et al. (2001) and Rasco-Gaunt et al. (2001) and fully described by Sparks and Jones (2004). Juvenile plants that survived selection were transferred to soil and grown to maturity.

2.4. Southern blotting

Genomic DNA was extracted from leaf tissue using the CTAB method following Stacey and Isaac (1994) and was completely digested by *Bam*HI that cuts the plasmid used for transformation (pLRPT-R853) once at the 3′ end of the coding region. Plasmid DNA was completely digested with *Stu*I and *Bam*HI which excises the coding region. Genomic (10 μg) and plasmid (5 pg) DNA were separated by electrophoresis on a 0.9% (w/v) agarose gel at 20 V for ∼40 h and transferred by capillary blotting onto positively charged nylon membrane (Roche Diagnostics GmbH, Lewes, East Sussex, UK) using the method of Sambrook et al. (1989). Blotting, hybridisation and chemiluminescent detection were carried out as described in the DIG System User's Guide for Filter Hybridisation (Roche, Welwyn Garden City, UK). The filter was probed with DIG-labelled 452 bp probe generated by PCR using primers designed to the 5′ coding region of pLRPT-Dx5-R853.

2.5. Functional analysis of transgenic grain

The five transgenic lines and two control lines were grown in a random block experiment in the containment glasshouse at Long Ashton Research Station (North Somerset, UK) with 10 pots of each line and each pot containing five plants. Seeds from the replicate pots (ranging from 121 to 207 g/line) were bulked for analysis.

2.6. Milling

Initial grain protein and moisture content were measured using a Foss systems 6500 NIR spectrometer (Silver Spring, MD, USA) before overnight conditioning. The grain was

milled to flour in a Brabender Quadramat Junior mill, and moisture and protein was determined as above.

2.7. Mixing properties

A 2-g Mixograph (Rath et al., 1990) was used to evaluate the functional dough properties; the water absorption was estimated by Approved Methods (AACC, 1995) using the protein and moisture contents of the flour. Mixing was performed in duplicate and the following parameters were measured: mean time to peak dough development (mixing time, MT [s]), height of Mixograph trace at peak resistance (PR [a.u.]) and percentage decrease in dough resistance 3 min after the peak (resistance breakdown, RBD [%]).

2.8. HPLC analysis

SE-HPLC analysis was carried out to determine the polymeric and monomeric distribution of the flour gluten proteins; the overall size distribution of these proteins was described by the percentage unextractable polymeric protein (%UPP) (Larroque and Békés, 2000).

Quantification of the HMW gluteinin subunits was carried out using RP-HPLC following sequential extraction of the gliadins and glutenins (Marchylo et al., 1989) as modified by Larroque et al. (2001).

2.9. SDS-PAGE and gel scanning

Total proteins were extracted from flour samples and separated by SDS-PAGE using a Laemmli (1970) system as described by Shewry et al. (1995).

Protein were quantified on three replicate separations using *phoretix*™ software (Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK).

3. Results and discussion

3.1. Production and characterisation of transgenic lines

Wheat HMW subunit 1Dx5 comprises 827 amino acid residues (M_r 88,137) including a repetitive domain of 696 residues. This repetitive domain was extended by 157 residues (to 853) by duplication of the sequence corresponding to residues 228–384 (D'Ovidio et al., 1997). The resulting protein, called 1Dx5 R853, comprised 984 residues (M_r 104,583).

The gene encoding this modified protein was used to transform wheat line L88-31, which expresses only two HMW subunit genes (1Bx17 + 1By18). Four transformed lines were selected on the basis of expression of the HMW subunit transgene in their seed (as revealed by SDS-PAGE) and F3 generation seed were grown in a replicate pot experiment with lines B72-8-12.4 (also F3), L88-6 and L88-31 as controls. B72-8-12.4 is a transgenic line expressing the wild type 1Dx5 protein in the L88-31 background and was produced in the study reported by Barro et al. (1997). L88-6 is a near-isogenic relative of L88-31 but expresses genes for five HMW subunits: 1Ax1, 1Dx5, 1Dy10, 1Bx17 and 1By18 (Lawrence et al., 1988).

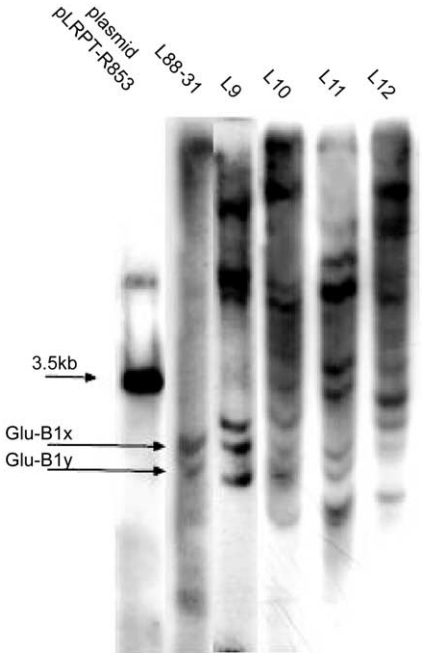

Fig. 1. Southern blot of plasmid (lane 1), and genomic DNA probed with a 452 bp, DIG-labelled fragment of pLRPT-R853 coding sequence. Plasmid DNA was digested with *Stu*I and *Bam*HI to release the coding sequence. Genomic DNA was cut once at the 3′ end of the coding region by *Bam*HI.

Southern analysis confirmed that the four lines (L9–L12) had originated from independent transformation events (Fig. 1). Multiple hybridising bands indicated that all four lines contained complex integration events with between four and more than eight transgene copies present. The two hybridising bands indicated in the L88-31 host genotype are presumed to correspond to the Glu-1Bx and Glu-1By genes (based on Harberd et al., 1986).

SDS-PAGE of total protein extracts from white flour fractions showed that three of the transgenic lines (L10-3-7, L12-3-1 and L11-1-7, Fig. 2, tracks a–c) contained additional HMW subunit bands of the mobility expected for 1Dx5 R853. These bands also co-migrated with recombinant 1Dx5 R853 protein expressed in *E. coli* (D'Ovidio et al., 1997) (results not shown). However, in one of the transgenic lines (L9-2-5, Fig. 2, track d), the additional protein had greater mobility than expected. It is probable that this band corresponds to a truncated form of 1Dx5 R853. Such rearranged forms of HMW subunit occur quite commonly in transgenic wheat lines (Barro et al., 1997) and are thought to result from recombination within the nucleotide sequences encoding the repeated peptide motifs. Similar mechanisms and events may also have contributed to the evolution of the naturally occurring subunit alleles (D'Ovidio et al., 1996).

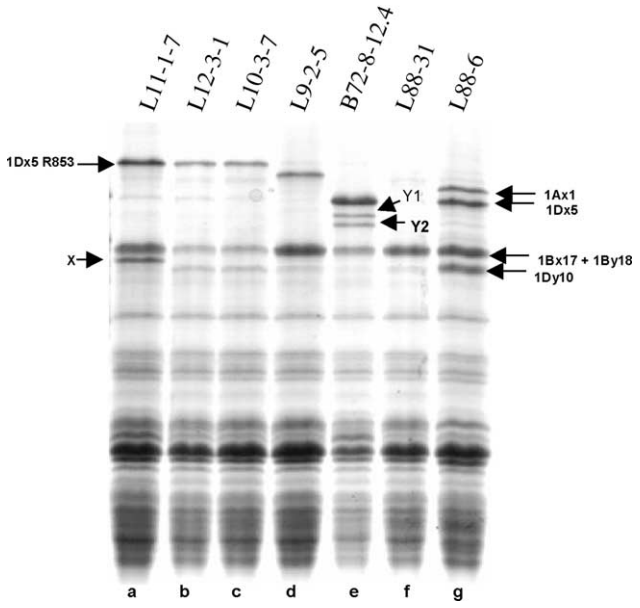

Fig. 2. SDS-PAGE of total seed proteins extracted from flour of control (tracks f, g) and transgenic (tracks a–e) lines. The arrows indicate HMW subunit bands discussed in the text.

Truncated HMW subunits also appeared to be present in two of the lines expressing the subunits of the expected mobility. Thus, the 'control' transgenic line B72-8-12.4 contained two bands of slightly faster mobility in addition to the subunit 1Dx5 encoded by the transgene (these are labelled y1 and y2 in Fig. 2, track e), while line L11-1-7 contained an additional band of slightly greater mobility than subunits 1Bx17 + 1By18 (labelled x in Fig. 2, track a).

The proportions of the total and individual HMW subunits in the flour samples were determined by quantitative scanning of SDS-PAGE gels (Table 1).

It is clear that the expression of the additional HMW subunits resulted in substantial increases in the total proportion of HMW subunits in lines L11-1-7, L9-2-5 and B72-8-12.4, with the latter approaching the proportion present in L88-6. The additional truncated 1Dx5 bands accounted for 4.06% of the total protein in L11-1-7 and 4.65% in B72-8-12.4 and presumably contributed to the effects on %UPP and mixing properties observed in these lines (see below). In contrast, despite expressing the transgenes, the combined proportions of HMW subunits in L10-3-7 and L12-3-1 were lower than in the control line L88-31. This is because the expression of the transgenes was associated with reduced expression of the endogenous chromosome 1B-encoded subunits, from about 7.6% of the total protein in L88-31 to about 4% in L10-3-7 and 3.4% in L12-3-1 (Table 1). It should also be noted that the proportion of HMW subunits determined for L88-6 (17.7%) is substantially higher than reported previously for the same line by Rooke et al. (1999) (12.7%). This probably resulted from differences in the material used, which was grown in a separate glasshouse experiment, and in the precise methods used for quantitation. Similar differences in the material and methods may also account

Table 1
Characteristics of the control and transgenic lines

	Endogenous HMW subunits present	Flour protein content (%)	Transgene products present	% Total protein present in HMW subunits			
				1Bx17 + 1By18	1Dx major bands	1Dx additional bands	Total HMW
L88-31	17, 18	11.6	–	7.60 ± 0.19	–	–	7.6 ± 0.19
L88-6	1, 5, 10,17, 18	11.4	–	5.84 ± 0.14	4.40 ± 0.092	–	17.69 ± 0.25
L10-3-7	17, 18	10.7	1Dx5 R853	4.02 ± 0.43	2.51 ± 0.25	–	6.53 ± 0.67
L11-1-7	17, 18	12.2	1Dx5 R853	5.66 ± 0.046	4.01 ± 0.026	4.06[a] ± 0.047	13.74 ± 0.047
L12-3-1	17, 18	10.8	1Dx5 R853	3.37 ± 0.13	2.16 ± 0.007	–	5.53 ± 0.12
L9-2-5	17, 18	11.4	1Dx5 R853 truncated	8.15 ± 0.36	3.07 ± 0.13	–	11.22 ± 0.49
B72-8-12.4	17, 18	10.5	1Dx5	4.57 ± 0.049	7.39 ± 0.12	4.65[b] ± 0.13	16.61 ± 0.27

	SE-HPLC			Mixograph parameters		
	%UPP[c]	Glu:Gli[d]	Poly:mono[e]	MT	PR	RBD
L88-31	19.9	1.22	0.96	178	485	9.5
L88-6	52.19	1.35	1.00	281	777	6.0
L10-3-7	19.83	1.18	0.91	109	385	16.5
L11-1-7	37.8	1.32	0.99	354	483	3.5
L12-3-1	16.8	1.27	0.95	111	381	17.5
L9-2-5	33.9	1.28	0.97	266	399	7.0
B72-8-12.4	30.9	1.46	1.04	186	223	3.5

[a] Band x.
[b] Bands Y1 + Y2.
[c] % Unextracted polymeric protein.
[d] Glutenin:gliadin ratio.
[e] Polymer:monomer ratio.

for the fact that the Mixograph properties of the two samples of L88-6 (see below) were not identical.

The effects of the transgenes on the proportions of glutenin proteins and insoluble high molecular mass glutenin polymers (called unextracted polymeric proteins, UPP) were determined by sequential extraction and RP-HPLC. The most striking difference was in the %UPP, which was higher in L88-6 (52%) and three transgenics L11-1-7 (38%), L9-2-5 (34%) and B72-8-12.4 (31%) than in L88-31, L10-3-7 and L12-3-1 (all below 20%).

3.2. Effects of transgenes on mixing properties

The protein contents of white flours milled from the lines ranged from 10.5% (B72-8-12.4) to 12.2% (L11-1-7) (Table 1).

The effects of the transgenes on dough mixing properties were determined using a 2 g Mixograph. The Mixograph records the increase in stress as dough is mixed to its maximum resistance and the subsequent decrease in stress on overmixing. A number of measurements are taken that relate to dough strength and stability of which three are used most widely. These are the mixing time (MT, measured in seconds), peak resistance (PR, in arbitrary units) which positively correlated with dough strength and resistance breakdown (RBD, also in arbitrary units) which measures the stability of the dough to overmixing. Typical Mixograph curves for all lines, except L10-3-7 (which was essentially the same as L12-3-1), are shown in Fig. 3 and the major parameters summarised in Table 1.

Analysis of the lines expressing 1Dx5 R853 showed different effects. L10-3-7 (not shown) and L12-3-1 (Fig. 3d) showed similar Mixograph profiles, with shorter mixing times than L88-31 (109 and 111 s, respectively, compared with 178 s) and lower PR (381 and 385 compared with 485). In these lines the dough strength was clearly lower than in L88-31, but there was no evidence for the highly detrimental effects observed with the wild type 1Dx5 transgene. L9-2-5, expressing the truncated form of 1Dx5 R853, had a similar mixing curve to L88-31, with a greater mixing time (266 s) and lower PR (399) (Fig. 3e) while in L11-1-7 (expressing the normal form of 1Dx5 R853) the MT was also increased (to 354 s) but there was no effect on PR (483) (Fig. 3f). These results indicate that both lines had increased dough strength but lacked the detrimental effects associated with the normal length 1Dx5 subunit. It is notable that L11-1-7 had a much higher level of transgene expression (over 8%) than L12-3-1 and L10-3-7 (2.16 and 2.51%, respectively) and

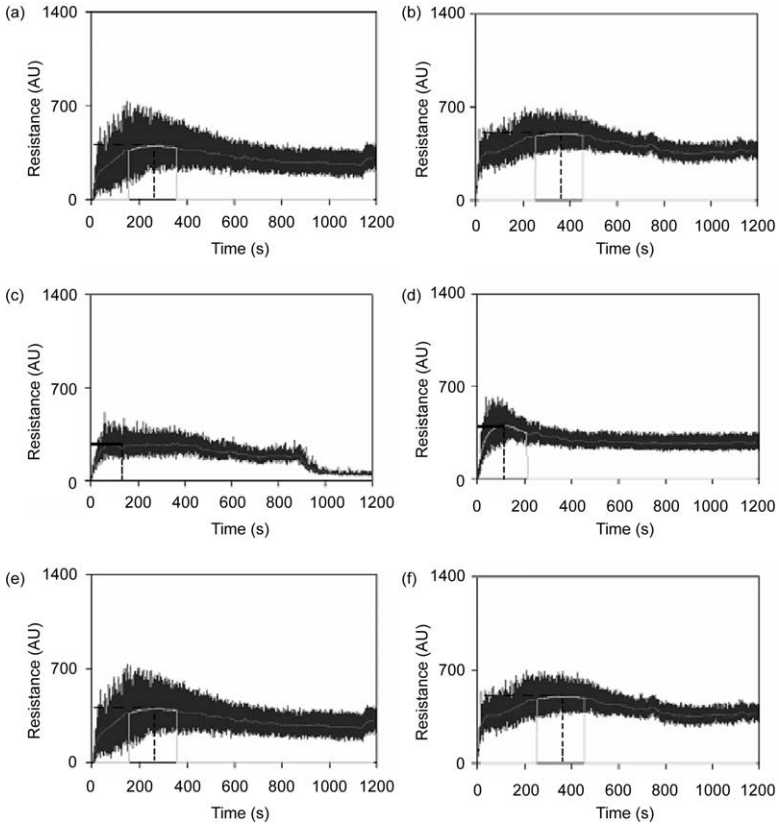

Fig. 3. Mixograph profiles of doughs of (a) L88-6; (b) L88-31; (c) B72-8-12.4; (d) L12-3-1; (e) L9-2-5 and (f) L11-1-17.

that the level of expression in L9-2-5 was also slightly higher (approximately 3%). In addition, L11-1-7 and L9-2-5 both contained higher proportions of unextractable glutenin polymers (UPP in Table 1): over 30% compared with 20% or less in L88-31, L10-3-7 and L12-3-1. The resistance breakdown (RBD) was also decreased in L11-1-7, L9-2-5 and B72-8-12.4 but was increased in the other transgenics. It is particularly noteworthy that the significant decrease in RBD in L11-1-7 was not associated with the detrimental effects on the mixing curve observed in B72-8-12.4.

4. Discussion

Comparison of the transgenic and control lines showed a clear relationship between the total amount of HMW subunits (expressed as % total protein) and the % UPP, which ranged from below 20% in L88-31, L10-3-7 and L12-3-1 (all of which contained less than 10% HMW subunits) to a maximum of 52.19% in L88-6 (17.7% total HMW subunits). These differences, which in the transgenics probably resulted from a combination of variation in transgene expression levels and co-suppression, meant that it was not possible to relate differences in % UPP to the specific HMW subunit composition. Lines L11-1-7 and L9-2-5 also showed increases in mixing time compared to L88-6 but peak resistance was either not affected (L11-1-7) or decreased (L9-2-5). In contrast, line B72-8-12.4 showed the characteristic negative effect on dough mixing which has previously been reported when subunit 1Dx5 was expressed in bread or pasta wheats (He et al., 1999; Popineau et al., 2001; Rooke et al., 1999). The reasons for this detrimental effect are not known but it has been suggested that the additional cysteine residue present in the repetitive domain of subunit 1Dx5 compared with other x-type HMW subunits results in the formation of highly cross-linked glutenin polymers which are unable to hydrate fully when water is added and mixed to form dough (Popineau et al., 2001). Butow et al. (2003) have also presented evidence that a precise balance of x:y subunits is required, showing that the detrimental effects of high level expression of the 1Dx5 transgene on dough mixing can be 'titrated out' by the incorporation of increasing amounts of subunit 1Dy10 purified from grain. However, this effect is clearly restricted to subunit 1Dx5 as the expression of subunit 1Ax at the same levels and in the same backgrounds had the expected positive effect on dough strength (He et al., 1999; Popineau et al., 2001). The fact that the effect associated with 1Dx5 was not observed in any of the transgenic lines expressing either 1Dx5 R853 or truncated forms of this subunit may indicate that this subunit does not promote the formation of such highly cross-linked polymers as the wild type 1Dx5 subunit. This difference in behaviour could result from differences in the expression levels of the 1Dx5 and 1Dx5R853 subunits in the transgenic lines and/or their ability to form disulphide bonds. In particular, the extended length of the repetitive domain in the 1Dx5R853 subunit could restrict the ability of the cysteine residue present in this domain to form the inter-chain disulphide bonds that are considered to be responsible for the unusual mixing properties associated with lines expressing the 1Dx5 transgene (Popineau et al., 2001).

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